J Wood Sci (1999) 45:53–63

ORIGINAL ARTICLE

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Antioxidative activity of tree phenolic constituents I: Radical-capturing reaction of flavon-3-ols with radical initiator

Received January 27, 1998 / Accepted June 5, 1998

Abstract The present work was undertaken from the standpoint of radical-capturing ability with regard to the antioxidative ability of flavonoids, especially flavonols distributed widely in woody plants. In regard to the flavonols, six methyl derivatives were initially prepared from quercetin and its litinoside. Their radical-capturing constants were determined strictly by the stopped-flow spectroscopic method. It was proved that the radical-capturing ability of quercetin mainly involves the vicinal $C_{3'}$ and $C_{4'}$ hydroxyl groups and the C_3 hydroxyl group. To clarify the reaction mechanism begun at the C_3 hydroxyl group of quercetin, 5,7,3′,4′-tetramethylquercetin (TMQ), flavon-3-ol (F3O) and so on were treated with 2,2′-azo-bis-(2,4-dimethylvaleronitrile) (AMVN). Six products (1–6) containing one depside and its two hydrolytic products, two valeronitrile adducts, and others were isolated from the reaction mixture of TMQ and their structures determined by instrumental analyses. Similarly, F3O gave four products, 7–10, which corresponded to the above products 1–3 and 5 (one depside, its two hydrolytic products, and one adduct), respectively. 3,5,7,3′,4′-Pentamethylquercetin (PMQ) and flavon-3-*O*-methylate (F3M) gave no products. The quantitative change of the products with reaction time was determined spectroscopically. An initial reaction pathway for the radical-capturing reaction of flavon-3-ols with AMVN was proposed based on the products and their amounts. The main route – formation of depside and its hydrolytic products via ketohydroperoxide (3″) or ketohydroperoxy radical (4″) – was similar to that of the oxidation reaction of quercetin with quercetinase and light.

Key words Antioxidative activity · Radical-capturing ability · Flavonol · 2,2′-Azo-bis-(2,4-dimethylvaleronitrile) (AMVN) · Reaction mechanism

Introduction

Studies on the antioxidative activity of natural products, which are of interest to the medical, pharmaceutical, and food industries, are undertaken based on accumulated experimental and epidemiological evidence suggesting the involvement of free radical-mediated oxidation of lipids in a variety of diseases, carcinogenesis, and aging.[1,2] Such approaches are becoming popular among researchers in various fields,[3–5] and many results have already been reported in the literature.[6–15] We are interested in the activity of woody plant phenolic constituents for the effective and useful use of woody plant biomass resources. The antioxidative activities of more than 90 natural constituents, such as stilbenes, flavonoids, diarylbutanoids, and other phenolics, have been evaluated in regard to their peroxide value and radical-capturing ability.[16–18] Consequently, many promising phenolic constituents containing flavonoids have been discovered.[18] Furthermore, the relation between activity and chemical structure have been discussed, though there was a limit owing to samples synthesized enzymatically.[18]

In general, many of flavonoids are known to have remarkable antioxidative ability, and the relation between antioxidative activity and the structure of the flavonoids has been discussed.[6,8–10,19] For example, it was recognized that (1) the hydroxyl group was required for the demonstration of antioxidative activity of flavonoids, (2) increasing the hydroxyl group numbers tended to increase activity, and (3) all flavonoids with vicinal hydroxyl groups, such as the $C_{3'}$,$C_{4'}$-dihydroxylated configuration, demonstrated strong antioxidative activity.[8] The degree of antioxidative activity of flavonoids concerning the C ring structure has not yet

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Part of this paper was presented at the 46th and 47th annual meetings of the Japan Wood Research Society, Kumamoto and Kouchi, April 1996 and 1997

been definitively defined,[20,21] and establishment of definite comparison methods for determing antioxidative activity of various natural constituents is desirable. Generally, it has been difficult to show the relation of strength and weakness of the activity of phenolic constituents because the results have differed with each test method.[22,23] Our results with various natural flavonoids based on their peroxide value and radical-scavenging ability have also been ambiguous.[18]

Recently, a stopped-flow spectroscopic method was noted among scientists in a wide range of research fields to be a test that can be applied for this purpose.[24,25] We had investigated and established a method that can evaluate the antioxidative activity strictly by index number.[26,27] A comparison of radical-capturing constants has been carried out on five typical natural flavonoids with one oxygenation pattern at the A and B rings (5,7,3′,4′-tetrahyroxylated structure). The constants ($M^{-1}s^{-1}$) decreased in the following order: quercetin (flavonol) (1141) > luteolin (flavone) (297) > *d*-catechin (flavan-3-ol) (70) > eriodictyol (flavanone) (43) > taxifolin (flavanonol) (28). This tendency was supported by a comparison of flavonoids with a 5,7,4′-trihydroxylated structure, except flavan-3-ol. Consequently, it was revealed that quercetin (5,7,3′,4′-tetrahydroxyflavonol) had the strongest ability and was a remarkably good target compound for further work, because it is widely distributed in woody plants.[28,29] The detailed results by the stopped flow method will be reported in the near future.

Different from aliphatic constituents such as α-tocopherol, the antioxidative reaction mechanism of aromatic constituents such as quercetin has not been definitely determined so far, though a few studies on flavonoids and others have been initiated.[30–32]

The aim of this work is to show the process of the radical-capturing reaction, which starts at the C_3 hydroxyl group in flavonols (e.g., quercetin). We have tried to establish how flavonoids have adapted to this compound.

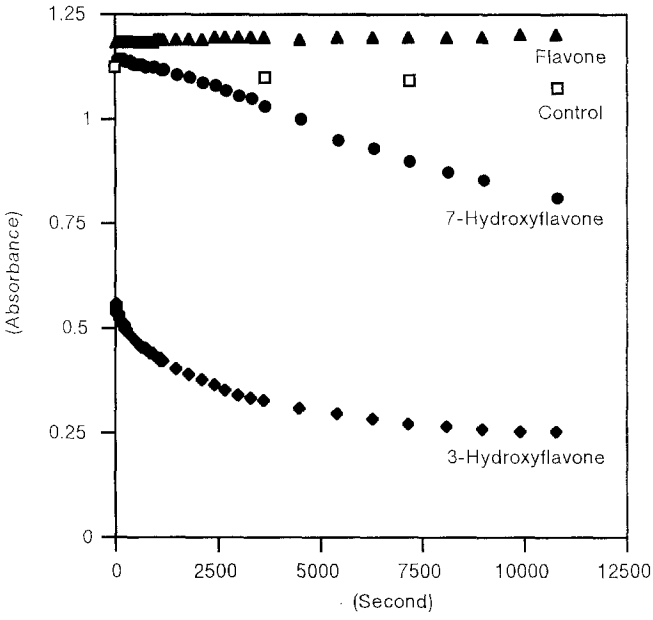
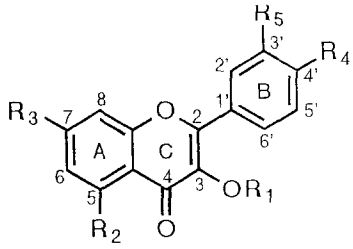


Fig. 1. Radical-capturing abilities of simple flavonoids. Specimen concentration 1.0×10^{-3} M; radical initiator (DPPH) concentration 5.0×10^{-4} M



Compound	I	R_1:H; R_2,R_3,R_4,R_5:OCH_3
	II	R_1:H; R_2:OH; R_3,R_4,R_5:OCH_3
	III	R_1:H; R_2,R_5:OH; R_3,R_4:OCH_3
	IV	R_1:CH_3; R_2,R_3:OCH_3; R_4,R_5:OH
	V	R_1:CH_3; R_2,R_3,R_4,R_5:OCH_3
	VI	R_1:CH_3; R_2:OH; R_3,R_4,R_5:OCH_3
	VII	R_1,R_2,R_3,R_4,R_5:H
	VIII	R_1:CH_3; R_2,R_3,R_4,R_5:H

Fig. 2. Compounds I–VIII

Results and discussion

Strict comparisons of the radical-capturing ability of quercetin methyl derivatives

The antioxidative activity and its reactivity of quercetin and its derivatives were considered in detail from the standpoint of radical-capturing ability. Prior to the main experiment, the radical-capturing ability and the reaction processes of simple flavonoid compounds were compared by the usual spectroscpic method using the radical initiator 1,1-diphenyl-2-picrylhydrazil (DPPH).[33] Figure 1 shows that the hydroxyl group, not the flavonoid skeleton, is necessary for radical-capturing. The degree of contribution of the C_3 hydroxyl group was greater than that of the C_7 hydroxyl group.

Next, quercetin methyl derivatives I–VI were prepared from quercetin or its rutinoside (rutin), with a slight difference in reactivity of their hydroxyl groups (Fig. 2). The structures of the derivatives were confirmed by instrumental analyses.

The radical-capturing ability, reaction process, and radical-capturing constant of quercetin methyl derivatives were determined to establish the different reactivities of their hydroxyl groups (Fig. 3, Table 1). The strong reactivity due to the vicinal $C_{3'}$ and $C_{4'}$ dihydroxyl groups on the B ring was confirmed in the experiment using 3,5,7-trimethylquercetin (IV). The considerable reactivity of the C_3 hydroxyl group was shown in the experiment using 5,7,3′,4′-tetramethylquercetin (TMQ) (I). The experiment with 3,7,3′,4′-tetramethylquercetin (VI) showed the very

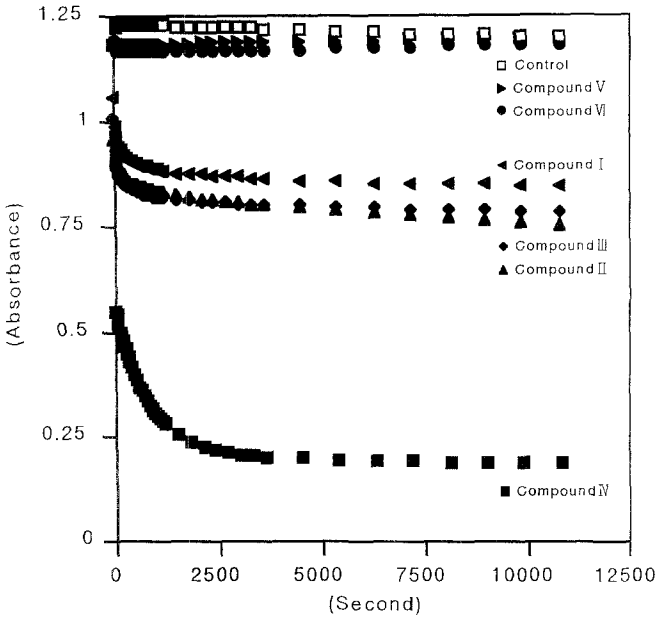


Fig. 3. Radical-capturing abilities of quercetin methyl ethers. Specimen concentration 1.0×10^{-3} M; radical initiator (DPPH) concentration 5.0×10^{-4} M

Table 1. Radical capturing constants of quercetin methyl ethers by stopped-flow spectroscopy.

Substrate	Radical-capturing constant ($M^{-1}s^{-1}$)
Compound I (5,7,3′,4′-tetramethylquercetin)	47
Compound II (7,3′,4′-trimethylquercetin)	104
Compound III (7,4′-dimethylquercetin)	91
Compound IV (3,5,7-trimethylquercetin)	3980
Compound V (3,5,7,3′,4′-pentamethylquercetin)	Not calculated[a]
Compound VI (3,7,3′,4′-tetramethylquercetin)	Not calculated[a]
Compound VIII (falvon-3-ol)	34

Substrate concentration 1.0×10^{-3} M; radical initiator (DPPH) concentration 5.0×10^{-5} M.
[a] Could not calculated the constant because of the low reactivity or no reactivity of the substrate

weak reactivity of the C_5 hydroxyl group. Furthermore, in experiments with 7,3′,4′-trimethylquercetin (II) and 7,4′-dimethylquercetin (III), which are thought to be derivatives of TMQ, the reactivities were not enlarged beyond our expectation, which meant that the C_5 and $C_{3'}$ hydroxyl groups contributed little. These results suggested that it was mainly the two pairs of the vicinal $C_{3'}$ and $C_{4'}$ and C_3 hydroxyl groups that contributed to the radical-capturing ability of quercetin. The reactivity of the $C_{4'}$ hydroxyl group could not be investigated in the present experiment, to our disappointment.

We believed that the antioxidative reaction of quercetin was complex owing to its plural hydroxyl groups. Thus it would be better to investigate every hydroxyl group when studying the antioxidative reaction mechanism of flavonol as quercetin. We first began to clarify the reaction pathway that starts from the C_3 hydroxyl group of quercetin.

Reaction of flavonol derivatives with AMVN and its products

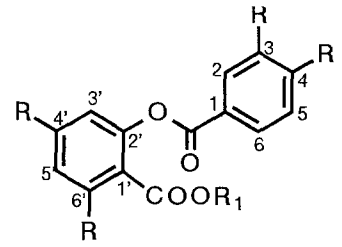
TMQ (I), together with flavon-3-ol (F3O) (VII), flavon-3-*O*-methylate (F3M) (VIII), and PMQ (V), was used in the experiment on the radical-capturing reaction mechanism.[34,35] In this part of the experiment 2,2-azo-bis-2,4-dimethylvarelonitrile (AMVN) radical initiator was used instead of 1,1-diphenyl-2-picrylhydrazil (DPPH) radical initiator because of its reactivity with flavonoid substrates and the isolation procedure of reaction products.

Reaction products from TMQ (I)

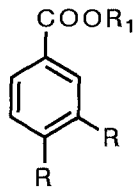
Six products (1–6) were isolated from the reaction mixture of the substrate (TMQ) (I) with AMVN. Their structures were identified or estimated as follows.

Product 1. Product 1′, a methyl derivative of product 1, was obtained from the acidic portion of the reaction mixture, followed by methylation. The ^{1}H-NMR spectrum of product 1′ showed the presence of four methoxyl groups, one esterified methyl group, and five aromatic protons, which amounted to 20 protons in total. Furthermore, five aromatic protons were divided into three protons belonging to the 1,3,4-trisubstituted aromatic ring and two protons to the 1,2,3,5-tetrasubstituted aromatic ring (Fig. 4). In the ^{13}C-NMR spectrum were one methyl group carbon of one ester moiety, four carbons of methoxyl groups, twelve carbons of two aromatic rings, and two carbons of carbonyl groups. Both carbonyl carbons were judged to be ester carbonyls based on their chemical shifts. Accordingly, the presence of at least eight oxygen atoms might be expected in the product. The molecular ion fragment (m/z 376) of DI-MS spectrum on product 1′ supported the estimated molecular formula $C_{19}H_{20}O_8$. Also, the mass spectrometry (MS) spectrum had characteristic fragment ions m/z 165, $C_6H_3(OCH_3)_2{\equiv}CO^+$ (A^+) and m/z 137, $C_6H_3(OCH_3)_2^+$ ($A{-}CO^+$). The structure of methyl-4′,6′-dimethoxysalicyloxy-3,4-dimethoxybenzoate (methyl-4,6-dimethoxysalicyloxy-veratrate) was estimated for product 1′. The structure was confirmed by direct comparison with 4′,6′-dimethoxysalicyloxy-3,4-dimethoxybenzoate, the authentic depside prepared photochemically from TMQ by Matsuuras' method following methylation.[36] Consequently, the final structure of product 1 became 4,6-dimethoxysalicyloxy-veratic acid. The corresponding depside was isolated from the antioxidative reaction mixture of quercetin itself by Hirose et al.[37]

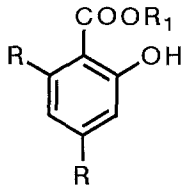
Products 2 and 3. The structures of methylated products 2′ and 3′ were determined spectrometrically. Methyl-3,4-



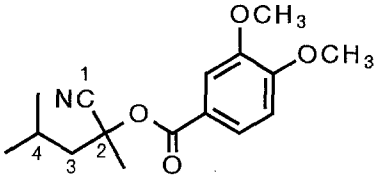
R:OCH_3; R1:H Product 1
R:OCH_3; R1:CH_3 Product 1'
R:H; R1:H Product 7
R:H; R1:CH_3 Product 7'



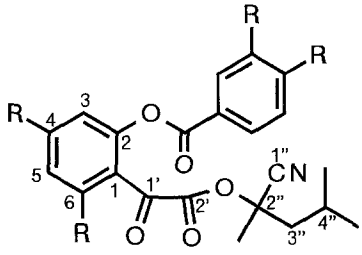
R:OCH_3; R1:H Product 2
R:OCH_3; R1:CH_3 Product 2'
R:H; R1:H Product 9
R:H; R1:CH_3 Product 9'



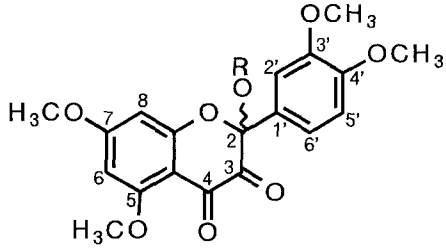
R:OCH_3; R1:H Product 3
R:OCH_3; R1:CH_3 Product 3'
R:H; R1:H Product 10
R:H; R1:CH_3 Product 10'



Product 4



R:OCH_3 Product 5
R:H Product 8



R:H Product 6
R:CH_3 Product 6'

Fig. 4. Products

dimethoxybenzoate (methyl veratrate) (2′) and methyl-2-hydroxy-4,6-dimethoxybenzoate (methyl-4,6-dimethoxysalicylate) (3′) have been identified. The final confirmation was undertaken by direct comparison with the authentic specimens prepared from purchased veratric and salicylic acids by methylation, TMQ by Matsuuras' method, or both.[36] Consequently, products 2 and 3 were veratric acid and 4,6-dimethoxysalicylic acid, which might be produced from the depside product 1 by a further hydrolytic reaction.

The isolation of products 1–3 indicated that the radical-capturing reaction of flavonols with AMVN, which began at the C_3 hydroxyl group, was similar to the photosensitized oxygenation reaction of flavonols in the presence of a catalytic amount of rose bengal, in regard to the formation of the depside and its degradative products.[36]

Product 4. According to the MS spectrum of product 4, its molecular ion m/z was 291, an odd number, and the presence of one nitrogen atom was suggested. The ^{1}H-NMR spectrum showed the presence of 12 protons consisting of one *gem*-methyl group, one *tert*-methyl group, one methylene group, and one methine group of 2,4-dimethylvaleronitrile moiety; six protons of two methoxyl groups; and three protons of the 1,3,4-trisubstituted aromatic ring. The ^{13}C-NMR spectrum revaled 16 carbons, with six of the nitrile moiety, six of aromatic rings, two of methoxyl groups, and one carbonyl group; the carbonyl carbon was found to be that of an ester. Thus product 4 seemed to have at least four oxygen atoms. This estimation was supported by the MS spectrum. The molecular ion, m/z 291, suggested a molecular formula of $C_{16}H_{21}O_4N$. Acccordingly, the results of instrument analysis indicated the presence of veratric acid moiety in product 4. Also, the veratrate moiety was linked by an ester bond to the C_2 position of 2,4-dimethylvaleronitrile moiety. Finally, 2- (2,4-dimethylvaleronitrile)-veratrate was estimated for the structure of product 4.

Product 5. Product 5 was characteristically positive using the thiophene condensation test and the imidazole formation test for the diketone structure of 1,2-dioxo compounds.[38] The MS spectrum was recorded, together with the characteristic fragment ion of A^+ (m/z 165), the molecular ion of m/z 499, which indicated the presence of nitrogen atom. ^{1}H-NMR spectroscopy recorded proton signals of the 1,3,4-trisubstituted aromatic ring as veratric acid moiety, aliphatics as 2,4-dimethylvarelonitrile moiety as above, and others. The others were assigned to six protons of two methoxyl groups and two aromatic protons suggesting a *meta* relation, respectively. The assignment of other protons supported the presence of the 1,2,4,6-tetrasubstituted aromatic ring. The presence of 26 carbon signals was confirmed on the ^{13}C-NMR spectrum. They were assigned to carbons of two aromatic rings (12 carbons), four methoxyl groups (4

carbons), the 2,4-dimethylvaleronitrile group (7 carbons), and three carbonyl groups (3 carbons). These carbonyl carbons could be divided into two ester carbonyl carbons and one ketone carbonyl carbon based on their chemical shifts. This estimation was supported by results of the above spot tests. Accordingly, the structure of product 5 was expected to be (2″,4″-dimethylvaleronitrile-2″-oxy)-1′-(1′,2′-diketoethyl)-4,6-dimethoxy-phenoxy-veratrate. The estimated structure did not contradict the results on H-H and H-C COSY spectral analyses.

Product 6. Prior to structural elucidation studies, product 6, was methylated to afford product 6′, thereby maintaining its stability. Product 6′ was positive to the thiophene condensation test and the imidazole formation test, which suggested a diketone structure.[38] The ^{1}H-NMR spectrum of product 6′ showed signals of one aliphatic methyl ester moiety, four methoxyl groups, a 1,3,4-trisubstituted aromatic ring, and a 1,2,3,5-tetrasubstituted aromatic ring (Fig. 4). Moreover, in the MS spectrum of product 6′ the characteristic fragment ions such as m/z 180 $[C_6H_2(OCH_3)_2(=O)(=CO)^+(B^+)]$, 152 $[B - CO]^+$, 165 (A^+), and 137 $[A - CO]^+$ – were detected, in addition to the molecular ion 388 (product 1). Consequently, the structure of product 6′ was estimated to be 2-methoxy-2-(3′,4′-dimethoxyphenyl)-5,7-dimethoxychroman-3,4-dione (2,5,7,3′,4′-pentamethoxyflavan-3,4-dione). The structure of product 6 was then estimated to be 2-hydroxy-5,7,3′,4′-tetramethoxyflavan-3,4-dione.

Reaction products from F3O (VII)

Two products (7′ and 8) were isolated from the reaction of the substrate F3O with AMVN. Their chemical structures, methyl salicyloxy-benzoate (7′) and (2″,4″-dimethylvaleronitrile-2″-oxy)-1′-(1′,2′-diketoethyl)-phenoxy-benzoate (8) were determined or estimated in a manner similar to that described above [see Reaction products from TMQ (I)]. On methylsalicyloxy-benzoate, the practice product was salicyl benzoic acid (7). Furthermore, the presence of two products, benzoic acid (9) and salicylic acid (10), which corresponded to products 2 and 3 from TMQ, was confirmed based on co-HPLC (high-performance liquid chromatography) with authentic specimens.

Reaction products from F3M (VIII) and PMQ (V)

Under the same reaction conditions as the above cases, the reactions of F3M (VIII) and PMQ (V) gave no reaction product. These results clearly indicated that the presence of a hydroxyl group at the C_3 position of the flavone structure was needed to start the radical-capturing reaction of the flavonols with a radical reagent. A similar phenomenon had been recognized with the photosensitized oxygenation of F3M.[36]

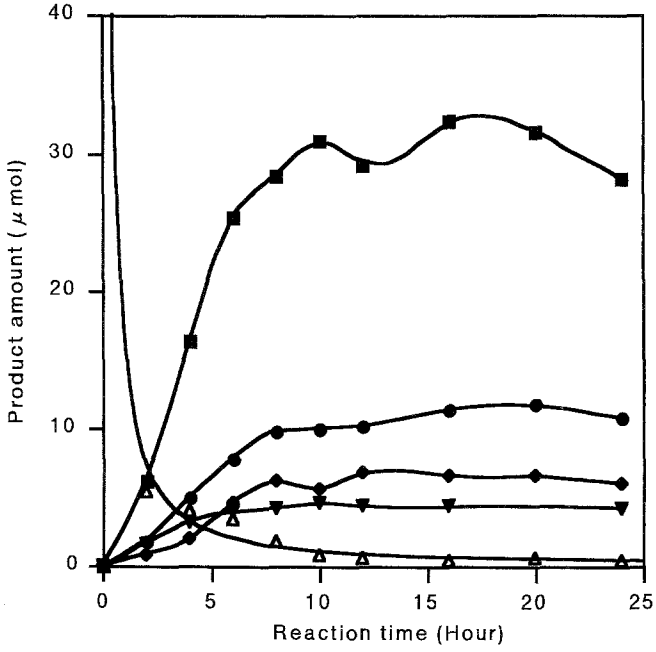


Fig. 5. Quantitative changes of the products during the reaction of 5,7,3′,4′-tetramethylquercetin with AMVN. Product 5 was determined directly, others were determined as methyl derivatives. *Squares*, product 1; *circles*, product 2; *diamonds*, product 3; *reverce triangles*, product 5; *open triangles*, substrate

Quantitative change of main products on reactions of TMQ (I) with AMVN

To determine the process of the radical-capturing reaction, the change of reaction products with time was investigated quantitatively using the reaction of TMQ (I) together with F3O (VII).

In the case of the TMQ (I) reaction, the substrate had almost disappeared at 10 h after the start of the reaction (Fig. 5). The depside formation reached its maximum at 15 h and maintained its production to the end of the experiment (24 h), though it had decreased a little at that stage. The other degraded products (2 and 3) were increased up to 8 h, and their levels were maintained up to the end. The pool size of product 3 (4,6-dimethoxysalicylic acid) always was smaller than that of product 2 (veratric acid). It was suggested that product 3, with one free hydroxyl group, might function as a substrate in the further radical-capturing reaction, though concrete data have not yet been obtained. Product 5, one of the adducts with 2,4-dimethylvaleronitrile radical, showed a tendency to vary similar to that of the depside (product 1), though the amount was small (one-seventh) (Fig. 5). These results indicated the presence of an interesting reaction mechanism of the flavonols.

In the reaction system of F3O (VII), the substrate disppeared 13 h after the start of the experiment (Fig. 6). The reactivity of this substrate was a little low in comparison with TMQ (I). The amount of depside (product 7) increased up to 4 h, with its level then maintained up the end of the reaction. The increase in the other main product (8)

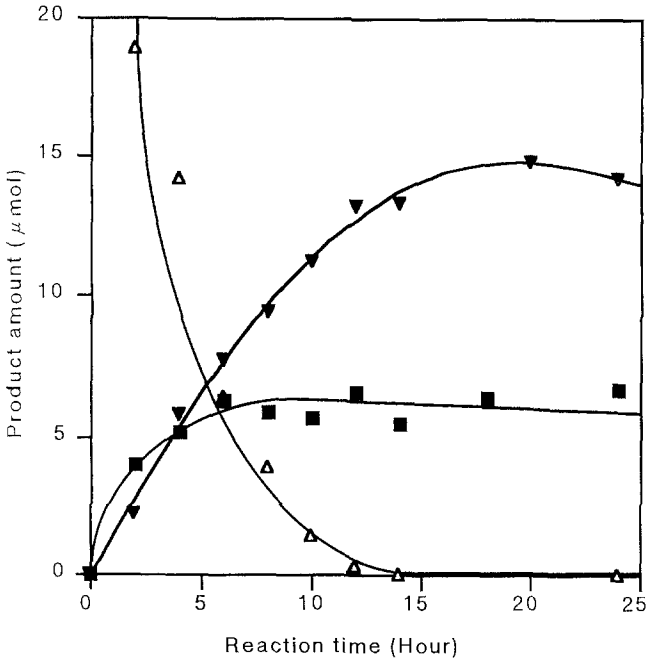


Fig. 6. Quantitative changes of the products after the reaction of flavon-3-ol with AMVN. Product 8 was determined directly. Product 7 and substrate were determined as methyl derivatives. *Open triangles*, substrate; *solid triangles*, product 8; *squares*, product 7

was allowed to continue for 13h, and the high level was maintained. These findings suggested that these products are moderately stable during the radical-capturing reaction, reflecting only one reaction site, the C_3 hydroxyl group of the flavonol. Thus further radical-capturing ability of these reaction products is weak in comparison with that of TMQ, possibly being influenced by the difference of electron numbers in the substrate molecule.

Proposal: the radical-capturing reaction pathway begins at the C_3 hydroxyl group of flavonols

Flavonoids with a C_3 hydroxyl group (e.g., TMQ and F3O) are radical-scavenging antioxidant substances, and they afford many reaction products. Their formation mechanisms are described below. On the proposed initial reaction pathway of flavonols with AMVN radical initiator, the initiator produced valeronitrile radicals, followed by formation of the peroxide radical (Fig. 7). In fact, singlet oxygen, which was formed by energy transfer from the triplet oxygen, might react with AMVN to give the AMVN radical.[39] The peroxide radical eliminated a proton radical from substrate I (or VII). The formed flavonol radical (1″) is attributed the formation of the substrate radical (2″). The substrate radicals accept one molecule of oxygen at the C_2 position and change it into the ketohydroperoxide radical (4″) via ketohydroperoxide (3″). For the radical (4″), two reaction routes might be considered. One (b) is conversion to product 1 via formation of intermediate (7″) by C_3 addition of the peroxide radical moiety in (4″). The other (a) is direct conversion to product 1 by the C_4 addition of the peroxide radical moiety in the intermediate (4″). According to quantitative studies, it is possible that the reactivity in the former route (b) is less than that in the latter (a). Furthermore, for formation of product 6, the other route, from the intermediate (4″), might be as described by Matsuura et al.[36]

The intermediate (6″) [or (5″)] changed into the depside product 1 (or 7), which was believed to be a stable compound, because the product was determined to be a main product. Furthermore, the depside was provide with two acid products 2 (or 9) and 3 (or 10) by hydrolytic degradation. In addition, two more reaction steps were seen with the valeronitrile radical addition: formation of adducts 4 and 5. The possibility on the formation of these products is small, though the products function further as substrate in the radical-capturing reaction.

Some of the reaction steps closely resembled the main steps of quercetin oxidation reactions catalyzed with dioxygenase (quercetinase) and light. This oxygenation was confirmed by an in vitro experiment of photosensitized oxygenation of flavonols in the presence of rose bengal, and the results have been discussed from the standpoint of the radical reaction.[36] Although the oxidative dimerized products had been isolated from the model experiment by Matsuura et al.,[36] such products have so far not been isolated from the present reaction mixture.

Isolation of further products from the reaction mixture of TMQ with AMVN is needed, as is their location on the reaction pathway, to delineate the detailed antioxidative reaction mechanism of flavonols. As the next stage of this research, we have begun to unravel the reaction mechanism that begins at the vicinal ($C_{3'}$ and $C_{4'}$ position) hydroxyl group of the flavonol when using 3,5,7-trimethylquercetin (IV). The results with be published in the near future. Nakayama et al. explained the radical-capturing reaction of (+)-catechin of flavan-3-ol and discussed the reaction products, which were produced owing to the presence of the vicinal hydroxyl group, and their possible formation mechanism.[40–42]

Experimental

The ^{1}H- and ^{13}C-NMR spectra were obtained with a Varian Inova 400 (400MHz) FT-NMR spectrometer. MS spectra were determined with a Shimadzu GCMS-QP 5000 mass spectrometer. HPLC was performed with a system of JASCO PU-980 pumps with JASCO UV-970 UV/VIS detector, using columns of Waters μ Bondasphere 5μ C_{18} 100 Å [3.9 (i.d.) × 150mm and 19.0 (i.d.) × 150mm]. The solvent systems were MeOH:M_2O = 7:3 and 6:4 v/v. The UV spectral data were recorded with Hitachi 100-20 and JASCO V550 UV/VIS spectrophotometers. Stopped-flow determination was undertaken on a Photal Otasuka electronics RA-401 stopped flow spectrophotometer equipped with a RA-451 data processing system [system disk: new version '88, 8, 20 (Photal Otsuka Electronics), MS-DS(TM)

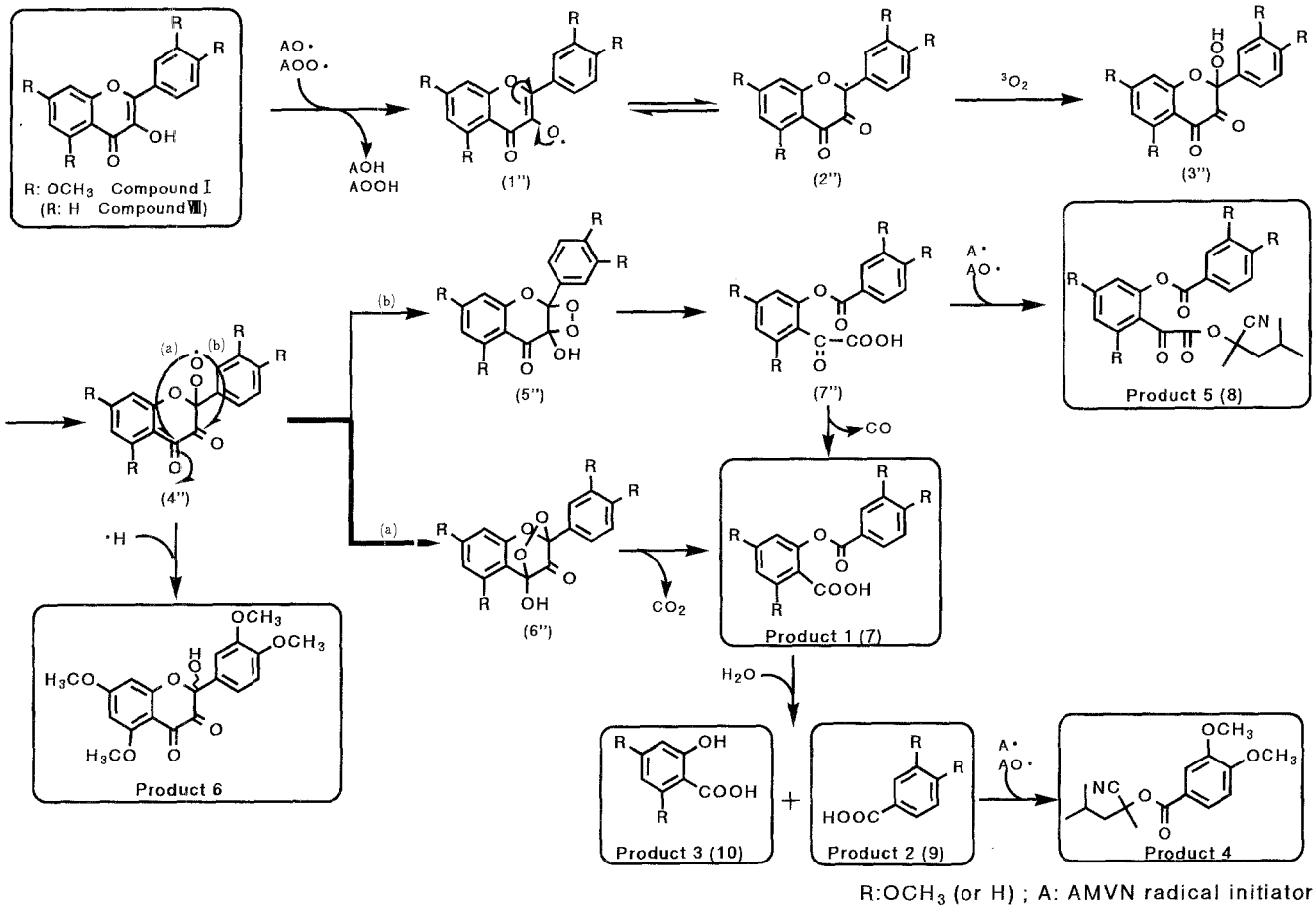


Fig. 7. Possible radical-capturing reaction mechanism of flavon-3-ols and AMVN

(Microsoft), and ASCII data conversion soft wave (Photal Otsuka Electronics)]. Thin-layer chromatography (TLC) and preparative TLC (PTLC) were conducted on silica gels (Merck, Kieselgel 60F254). Column chromatography (CC) was run on silica gels (Wako, Wakogel C-200 and/or Fuji Silysia, BW-200). For TLC and CC, the following solvent systems were used: chloroform ($CHCl_3$):acetone:benzene 9:1:0.5, and methylene chloride (CH_2Cl_2):acetone:benzene 9:1:0.5 v/v.

Materials

Flavonoids, flavon-3-ol, quercetin, and rutin were purchased. Quercetin methyl derivatives and flavon-3-*O*-methylate were prepared as below. Radical initiators of 1,1-diphenyl-2-picrylhydrazil (DPPH) and 2,2′-azo-bis-(2,4-dimethylvaleronitrile) (AMVN) were purchased.

Determination of radical-capturing ability by stopped-flow method

The DPPH ethanol (EtOH) solution (50 μM) was placed in the reservoir of the instrument and the definite sample EtOH solution (1 mM) was placed in the other resorvoir (see below). At 25°C, two solutions were introduced into the mixing apparatus, and the mixture was moved to the reaction vessel. At the reaction vessel, the changes in optical density were determined successively. The results were processed in a computer. Owing to the limits of the data-processing program, the sample concentrations was 20 times the DPPH radical concentration. Each result was based on three determinations.

Preparation of quercetin methyl derivatives

5,7,3′,4′-Tetramethylquercetin (TMQ) (I), 7,3′,4′-trimethylquercetin (II), 7,4′-dimetylquercetin (III)

Compound I was prepared from rutin (quercetin-3-*O*-β-rutinoside) by methylation with methyl iodide (CH_3I), followed by acid hydrolysis. In addition, compounds II and III were obtained from the hydrolyzed mixture as by-products and were purified by CC and PTLC.

Compound I (TMQ) DI-MS m/z (%): 358(100, M^+), 357(16.8), 330(1.7), 329(8.5), 312(52.2), 181(2.8), 179(23.3), 165(12.0), 163(16.1), and 149(10.7). ^{1}H—NMR ($CDCl_3$): δ

3.90, 3.93, 3.97, 4.00 (4 × 3H, each s, $C_{5,7,3',\&4'}$—OCH_3), 6.37 (1H, d, J = 2.20 Hz, C_6—H), 6.56 (1H, d, J = 2.20 Hz, C_8—H), 7.01 (1H, d, J = 9.15 Hz, $C_{5'}$—H), 7.80 (1H, d, J = 2.01 Hz, $C_{2'}$—H) and 7.83 (1H, d.d, J = 2.01 and 9.15 Hz, $C_{6'}$—H).

Compound II (7,3',4'-trimethylquercetin) DI-MS m/z (%): 344(100, M^+), 329(12.0), 301(16.6), 172(14.8), 167(4.7), 165(9.3), 157(9.3), 143(8.4), 135(11.1), and 69(8.6). ^{1}H—NMR ($CDCl_3$): δ 3.90, 3.98, 3.99 (3 × 3H, each s, $C_{7,3',\&4'}$—OCH_3), 6.39 (1H, d, J = 2.20 Hz, C_6—H), 6.50 (1H, d, J = 2.20 Hz, C_8—H), 7.01 (1H, d, J = 8.61 Hz, $C_{5'}$—H), 7.79 (1H, d, J = 2.01 Hz, $C_{2'}$—H), 7.84 (1H, d.d, J = 2.01 and 8.61 Hz, $C_{6'}$—H) and 11.73 (1H, s, $C_{5'}$—OH).

Compound III (7,4'-dimethylquercetin) DI-MS m/z (%): 330 (100, M^+), 315(37.9), 301(5.9), 287(11.2), 259(10.8), 167(4.1), 151(7.4), 149(19.0), 135(25.6), and 69(12.5). ^{1}H—NMR (as triacetate of compound III) ($CDCl_3$): δ 2.33, 2.36, 2.43 (3 × 3H, each s, $C_{3,5,\&3'}$—OAc), 3.90, 3.91 (2 × 3H, each s, $C_{7\&4'}$—OCH_3), 6.63 (1H, d, J = 2.38 Hz, C_6—H), 6.84 (1H, d, J = 2.38 Hz, C_8—H), 7.06 (1H, d, J = 8.61 Hz, $C_{5'}$—H), 7.55 (1H, d, J = 2.20 Hz, $C_{2'}$—H), and 7.73 (1H, d.d, J = 2.20 and 8.61 Hz, $C_{6'}$—H).

3,5,7-Trimethylquercetin (IV)

Quercetin was treated with α,α-dichlorodiphenyl methane, and the product was then methylated with CH_3I as described above, followed by deprotection. After purification by CC, compound IV was obtained.

Compound IV (3,5,7-trimethylquercetin) DI-MS m/z (%): 344(83.0, M^+), 343(100), 326(19.5), 297(12.9), 181(13.2), 158(42.3), 137(31.8), 128(17.5), and 109(13. 4). ^{1}H—NMR (as diacetate of compound IV) ($CDCl_3$): δ 2.33, 2.35(2 × 3H, each s, $C_{3',\&4'}$—OAc), 3.90, 3.91, 3.96(3 × 3H, each s, $C_{3,5,7}$—OCH_3), 6.34(1H, d, J = 2.20 Hz, C_6—H), 6.49(1H, d, J = 2.20 Hz, C_8—H), 7.33(1H, d, J = 8.61 Hz, $C_{5'}$—H), 7.96(1H, d, J = 2.01 Hz, $C_{2'}$—H), and 8.05(1H, d.d, J = 2.01 and 8.61 Hz, $C_{6'}$—H).

3,5,7,3',4'-PMQ (V) and 3,7,3',4'-tetramethylquercetin (VI)

Quercetin was methylated similarly. Compounds V and VI, as by-products, were isolated from the reaction mixture by CC.

Compound V (PMQ) DI-MS m/z (%): 372(100, M^+), 357(67.8), 343(8.4), 329(8.7), 329(8.7), 314(5.7), 181(13.0), 172(51.7), 165(14.9), 157(14.5), and 149(30.5). ^{1}H—NMR ($CDCl_3$): δ 3.88, 3.91, 3.97, 3.97, 3.97(5 × 3H, each s, $C_{3,5,7,3',\&4'}$—OCH_3), 6.34(1H, d, J = 2.20 Hz, C_6—H), 6.50(1H, d, J = 2.20 Hz, C_8—H), 6.98(1H, d, J = 9.16 Hz, $C_{5'}$—H), 7.68(1H, d, J = 2.20 Hz, $C_{2'}$—H), and 7.71(1H, d.d, J = 2.20 and 9.16 Hz, $C_{6'}$—H).

Compoud VI (3,7,3',4'-tetramethylquercetin) DI-MS m/z (%): 358(100, M^+), 343(46.5), 329(9.5), 327(14.5), 315(40.7), 167(10.3), 165(35.4), 163(14.5), and 135(13.6). ^{1}H—NMR ($CDCl_3$): δ 3.87, 3.89, 3.97, 3.98(4 × 3H, each s, $C_{3,7,3',\&4'}$—OCH_3), 6.36(1H, d, J = 2.20 Hz, C_6—H), 6.45(1H, d, J = 2.20 Hz, C_8—H), 7.00(1H, d, J = 8.42 Hz, H—$_{5'}$), 7.69(1H, d, J = 2.20 Hz, H—$_{2'}$), 7.74(1H, d.d, J = 2.20 and 8.42 Hz, H—$_{6'}$), and 12.65(1H, broad s, C_5—OH).

F3M (VIII)

Flavon-3-*O*-methylate (F3M) was prepared from F3O (VII) by the same methylation method as above.

F3M DI-MS m/z (%): 252(52.2, M^+), 251(100), 221(11.2), 181(10.5), 152(11.9), 125(10.1), 120(21.0), 105(14.4), 92(14.9), and 77(43.7). ^{1}H—NMR ($CDCl_3$): δ 3.90(3H, s, C_3—OCH_3), 7.41(1H, d.d.d, J = 1.10, 6.97 and 8.06 Hz, C_6—H), 7.54(2H, m, $C_{3'\&5'}$—H), 7.54(1H, m, $C_{4'}$—H), 7.54(1H, d.d, J = 1.10 and 8.54 Hz, C_8—H), 7.69(1H, d.d.d, J = 1.47, 6.96 and 8.49 Hz, C_7—H), 8.12 (2H, m, $C_{2'\&6'}$—H) & 8.28(1H, d.d, J = 1.47 and 8.06 Hz, C_5—H).

Reaction of flavonols with AMVN

A 1.0-mM aliquot of the substrate (TMQ) (I), F3O (VII), PMQ (V), or F3M (VIII) and 10 mM of AMVN radical initiator were dissolved in benzene (500 ml) and kept at 37°C for 12–14 h in the dark. The endpoint of the reaction was judged to be the disappearance of the substrate by monitoring with TLC. The reaction mixture was concentrated in vacuo to yield oily residue. The residue was dissolved in small amounts of EtOH. After keeping it at room temperature, the deposited AMVN was filtered off. The filtrate was again concentrated to give oils under reduced pressure. For the isolation, a part of the residue was methylated with diazomethane diethyl ether (Et_2O) solution. The methylated fraction was submitted to CC, PHPLC, and recrystallization for the isolation of products (see below).

Isolation and identification of reaction products

Reaction products of TMQ (I)

The methylated acidic portion from the reaction mixture was submitted to isolation procedures with CC, HPLC, PTLC, and recrystallization. Three products (1', 2', 3') were isolated and their structures determined. The specimens for final identification were prepared from 5,7,3',4'-tetramethylquercetin by the method of Matsuura et al.[36] and the methylation procedure (details were omitted).

Product 1' (methyl 4',6'-dimethoxysalicyloxy-3,4-dimethoxybenzoate) DI-MS m/z (%): 376(5.6, M^+), 345(0.9), 180(1.1), 165[100, $C_6H_3(OCH_3)_2$—CO^+) (A^+)], 149(1.5), 137(6.4, A−CO^+), 122(3.1), 107(2.2), and 77(6.6). ^{1}H—NMR ($CDCl_3$): δ 3.69(3H, s, $COOCH_3$), 3.83, 3.86, 3.96, 3.98(4 × 3H, each s, $C_{4,6,3',\&4'}$—OCH_3), 6.41(2H, s, $C_{3\&5}$—H), 6.95(1H, d, J = 8.42 Hz, $C_{5'}$—H), 7.64(1H, d, J = 2.01 Hz, $C_{2'}$—H), and 7.82(1H, d.d, J = 2.01 and 8.42 Hz, $C_{6'}$—H). ^{13}C—NMR ($CDCl_3$): δ 52.1($COOCH_3$), 55.6, 56.1, 56.1, 56.2($C_{4,6,3',\&4'}$—OCH_3), 96.9(C_3), 100.1(C_5), 109.4(C_1), 110.4($C_{2'}$), 112.3($C_{5'}$), 121.5($C_{1'}$), 124.5($C_{6'}$), 148.8,

151.1($C_{3'\&4'}$), 153.7, 159.5($C_{2\&6}$), 162.4(C_4), and 164.4, 165.1(2 × C=O).

Product 2′ (methyl 3,4-dimethoxybenzoate) DI-MS m/z (%): 196(94.0, M^+), 181(8.0), 165(100, A^+), 149(32.1), 137(23.0, $A-CO^+$), 135(19.5), 121(26.0), 105(39.8), 91(65.9), and 77(45.4). ^{1}H—NMR ($CDCl_3$): δ 3.89(3H, s, $COOCH_3$), 3.93, 3.93(2 × 3H, each s, $C_{3\&4}$—OCH_3), 6.83(1H, d, J = 8.42 Hz, C_5—H), 7.54(1H, d, J = 2.01 Hz, C_2—H), and 7.68(1H, d.d, J = 2.01 and 8.42 Hz, C_6—H).

Product 3′ (methyl 4,6-dimethoxysalicylate) DI-MS m/z (%): 212(28.0, M^+), 180[100, $C_6H_2(OCH_3)_2(=O)(=CO)^+)(B^+)$], 152(25.5, $B-CO^+$), 137(36.7), 123(5.7), 109(8.8), 105(8.0), 95(12.2), 91(16.4), and 69(30.4).^{1}H—NMR ($CDCl_3$): δ 3.82(3H, s, $COOCH_3$), 3.83, 3.92(2 × 3H, each s, $C_{4\&6}$—OCH_3), 5.97(1H, d, J = 2.38 Hz, C_5—H), 6.12(1H, d, J = 2.38 Hz, C_3—H), and 12.05(1H, s, C_2—OH).

The neutral fraction of the reaction mixture (substrate I) gave two products (4 and 5) by CC, PTLC, HPLC, or recrystallization.

Product 4 (2-, 2,4-dimethylvaleronitrile veratrate) DI-MS m/z (%): 291(19.7, M^+), 182(100, B^+), 180(2.9), 167(27,.0), 165(50.0), 153(0.3), 149(10.6), 139(9.9), 137(6.2), and 121(8.9). ^{1}H—NMR ($CDCl_3$): δ 1.08, 1.11[2 × 3H, each d, J = 6.23 Hz, $C_{4'}$—$(CH_3)_2$], 1.89(3H, s, $C_{2'}$—CH_3), 1.98–2.17(2H, m, $C_{3'}$—H_2), 1.98–21.7(1H, m, $C_{4'}$—H), 3.93, 3.95(2 × 3H, each s, $C_{3\&4}$–OCH_3), 6.90(1H, d, J = 8.42 Hz, C_5—H), 7.53(1H, d, J = 2.20 Hz, C_2—H), and 7.65(1H, d.d, J = 2.20 and 8.42 Hz, C_6—H). ^{13}C—NMR ($CDCl_3$): δ 23.3, 23.5, 23.6, 24.7[$C_{2'}$—CH_3, $C_{4'}$, $C_{4'}$—$(CH_3)_2$], 45.6($C_{2'}$). 52.0, 56.0($C_{3\&4}$—OCH_3), 80.4($C_{1'}$), 110.3(C_2), 111.9(C_5), 119.9($C_{(1)}N$), 122.6(C_1), 123.6(C_6), 148.6, 153.0($C_{3\&4}$), and 167.1(C=O).

Product 5 [(2″,4″-dimethylvaleronitrile-2″-oxy)-1′-(1′,2′-diketoethyl)-4,-4-dimethoxyphenoxy-veratrate]. Condensation test with thiophene and imidazole and imidazole formation test for 1,2-dioxo compound: positive.[38] DI-MS m/z (%): 499(0.9, M^+), 345(18.8), 182(4.0), 180(7.2, B^+), 165(100, A^+), 152(4.8), 137(9.6), 122(2.7), 107(1.9), 77(9.2), and 69(5.4). ^{1}H—NMR ($CDCl_3$): δ 0.99, 1.02[2 × 3H, d, J = 6.23 Hz, $C_{4''}$—$(CH_3)_2$], 1.83(3H, s, $C_{2''}$—CH_3), 1.92–2.00(2H, m, $C_{3''}$—H_2), 1.94—2.00(1H, m, $C_{4''}$—H), 3.89, 3.96, 3.96, 3.96(4 × 3H, each s, $C_{4',6',3,\&4}$—OCH_3), 6.42(1H, d, J = 2.20 Hz, C_5—H) 6.47(1H, d, J = 2.20 Hz, $C_{3'}$—H), 6.95(1H, d, J = 8.42 Hz, C_5—H), 7.65(1H, d, J = 2.20 Hz, C_2—H) & 7.84(1H, d.d, J = 2.20 and 8.42 Hz, C_6—H). ^{13}C—NMR ($CDCl_3$): δ 24.3, 24.7, 26.0, 26.2($C_{2''}$—CH_3, $C_{4''}$, and $C_{4''}$—$(CH_3)_2$, 49.0($C_{3''}$), 54.3, 56.9, 57.1($C_{4',6',3,\&4}$—OCH_3), 73.4($C_{2''}$), 97.5($C_{3'}$), 103.4($C_{5'}$), 110.5($C_{1'}$), 111.5(C_2), 113.6(C_5), 119.7($C_{(1)}N$), 122.5(C_1), 125.8(C_6), 149.8, 154.7, 155.2, 162.7, 163.7($C_{2',4'.6',3,\&4}$), 165.6(C=O), 166.9(C=O), and 182.5(C=O).

The remaining acidic part (of substrate I) was methylated with diazomethane solution. The methylated fraction was purified by CC, PTLC, PHPLC, or recrystallization to give product 6′ together with the above products.

Product 6′ (2-methoxy-5,7,3′,4′-tetramethoxyflavan-3,4-dione). Condensation test with thiophene and imidazole formation test: positive.[38] DI-MS m/z (%): 388(11.4, M^+), 223(6.2), 180(1.8, B^+), 167(1.2), 165(100, A^+), 152(4.0), 137(7.7), 122(2.9), 109(1.2), 94(1.9), and 77(7.2). ^{1}H—NMR ($CDCl_3$): δ 3.53(3H, s, C_2—OCH_3), 3.90, 3.91, 3.95, 3.96(4 × 3H, each s, $C_{5,7.3',\&4'}$—OCH_3), 6.04(1H, d, J = 1.83 Hz, C_6—H), 6.30(1H, d, J = 1.83 Hz, C_8—H), 6.93(1H, d, J = 8.6 Hz, $C_{5'}$—H), 7.82(1H, d, J = 2.01 Hz, $C_{2'}$—H), and 8.08(1H, d.d, J = 2.01 and 8.61 Hz, $C_{6'}$—H).

Reaction products of F3O (VII)

After evaporation of the reaction mixture of F3O, as described in earlier, the oily residue was dissolved in CH_2Cl_2, and the organic layer was washed 3 times with saturated $NaHCO_3$ solution. The combined $NaHCO_3$ layer was acidified with 1N HCl (pH 2), and extracted with CH_2Cl_2 (3 times). After washing with saturated NaCl solution and drying with anhydrous Na_2SO_4, the acidic organic layer was evaporated to give oils in vacuo. The acidic oils were methylated with diazomethane. The methylated fraction was submitted to PTLC, and one methylated product 7′ was isolated. The product 7′ was identified in comparison with methyl salicyloxybenzoate prepared photochemically from F3O by the method of Matsuura et al. following methylation.[36] After isolation of product 7′, the methylated fraction was submitted to co-HPLC analysis with authentic specimens, and methyl benzoate (9′) and methyl salicylate (10′) were detected. The formation of benzoic acid (9) and salicylic acid (10) was confirmed in the reaction mixture of F3O with AMVN.

Product 7′ (methyl salicyloxybenzoate) DI-MS m/z (%): 256(32.2 M^+), 225(1.7), 121(0.6), 120[1.0, $C_6H_4(=O)(C=O)^+(B'^+)$], 197(0.7), 105[100, $C_6H_5CO^+$ (A'^+)], and 77(32.6). ^{1}H—NMR ($CDCl_3$): δ 3.83(3H, s, OCH_3), 7.34(1H, d.d, J = 1.10 and 8.06 Hz, C_3—H), 7.45(1H, d.d.d, J = 1.10, 7.70, and 7.70 Hz, C_5—H), 7.77(4H, m, $C_{4,3',4',\&5'}$—H), 8.18(1H, d.d, J = 1.83 and 7.70 Hz, C_6—H) and 8.45(2H, m, $C_{2'\&6'}$—H). ^{13}C—NMR ($CDCl_3$): δ 52.5(OCH_3), 123.9(C_1), 124.4(C_3), 126.5(C_5), 129.0($C_{3'\&5'}$), 130.0($C_{1'}$), 130.7($C_{2'\&6'}$), 132.4(C_6), 134.0(C_4), 134.2($C_{4'}$), 151.3(C_2), and 165.5, 165.8(2 × C=O).

Products 9′ (methyl benzoate) and 10′ (methyl salicylate) PLC (MeOH:H_2O = 7:3 v/v) (retention time, minutes): product 9 (methyl benzoate) (9.7), product 10 (methyl salicylate) (12.7).

In the other case, the oily residue of the reaction mixture as mentioned above was washed 3 times with a mixture of Et_2O and CH_2Cl_2, and the washings were evaporated under reduced pressure. The residue was applied to CC, and product 8 was isolated.

Product 8 [(2″,4″-dimethylvaleronitrile-2′-oxy)-1′-(1′, 2′-diketoethyl)-phenoxy-benzoate] DI-MS m/z (%): 225(49), 213(+), 197(+), 185(+), 171(+), 149(+), 139(+), 121(5), 120(+, B^+), 106(8), 105(100, A^+), 94(5), 92(6), and 77(40). ^{1}H—NMR ($CDCl_3$): δ 0.98, 1.02 [2 × 3H, d, J = 6.23 Hz, $C_{4''}$—$(CH_3)_2$], 1.66(3H, s, $C_{2''}$—CH_3), 1.82–2.00(2H, m, $C_{3''}$—H_2), 2.00–2.16(1H, m, $C_{4''}$—H), 7.06–7.70(4H, m, $C_{3',4'5'\&6'}$—H), and 7.38–8.28(5H, m, $C_{2,3,4.5,\&6}$).

Reaction products of PMQ (V) and F3M (VIII)

Under reaction conditions similar to those for F3O (VII) and TMQ (I), two other substrates PMQ (V) and F3M (VIII) were reacted with AMVN, but they did not give any products.

Determination of variation on reaction products of TMQ (I) and F3O (VII) with AMVN

A mixture of TMQ (I) [or F3O (VII)] (0.1 mM) and AMVN (0.1 mM) in benzene (100 ml) was treated in the dark at 37°C. An aliquot of the reaction mixture was removed every 2 h for quantitative determination of products. The aliquot was concentrated under reduced pressure. Except for product 5 (also product 8) determination, the residue was methylated with diazomethane. The methylated product was dissolved in methanol (MeOH) containing an internal standard of hydroquinone Et_2O (based on the aliquot, 0.25 mg/ml). After filtration with DISMIC-13JP (PTFE 0.5 μm), the MeOH solution was subjected to HPLC analysis. Product 5 (8) was directly determined by HPLC after addition of the internal standard (Figs. 5, 6).

Acknowledgment This study was supported partly by a Grant-in-Aid for Scientific Research (07660212) from the Ministry of Education, Science, Sport and Culture of Japan.

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